

# Calmodulin kinase II accelerates L-type $\text{Ca}^{2+}$ current recovery from inactivation and compensates for the direct inhibitory effect of $[\text{Ca}^{2+}]_i$ in rat ventricular myocytes

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Some studies report that the positive relationship between L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca-L}}$ ) and frequency in cardiac myocytes is mainly due to a direct negative feedback of sarcoplasmic reticulum  $\text{Ca}^{2+}$  release on  $I_{\text{Ca-L}}$  inactivation while others provide evidence for activation of calmodulin kinase II (CaMKII). To further elucidate the role of endogenous CaMKII activity, the CaMKII inhibitory peptides, autocamtide-2 relating inhibitory peptide (AIP) and myristoylated AIP were applied using conventional and perforated patch-clamp methods. AIP inhibited the normal adaptive increase in  $I_{\text{Ca-L}}$  in response to abrupt increase in pacing frequency from 0.05 to 2 Hz. The positive  $I_{\text{Ca-L}}$ -frequency relationship was reversed by AIP and the inhibitory effect of AIP was significantly exaggerated at fast pacing rates. The onset of inactivation of  $I_{\text{Ca-L}}$  was not altered by AIP. After thapsigargin treatment, AIP slowed recovery from inactivation of  $I_{\text{Ca-L}}$  and this effect was exaggerated during fast pacing. Buffering of  $[\text{Ca}^{2+}]_i$  by BAPTA and EGTA accelerated recovery of  $I_{\text{Ca-L}}$  from inactivation, and BAPTA partly eliminated the effect of AIP on the recovery. We conclude that: (1)  $[\text{Ca}^{2+}]_i$  directly slows  $I_{\text{Ca-L}}$  recovery from inactivation; and (2)  $\text{Ca}^{2+}$ -dependent endogenous CaMKII activity accelerates the  $I_{\text{Ca-L}}$  recovery. Thus, at fast heart rates, elevated  $[\text{Ca}^{2+}]_i$  activates endogenous CaMKII and compensates for its direct inhibitory effect on  $I_{\text{Ca-L}}$  recovery from inactivation. Dynamic activity of endogenous CaMKII enhances the positive  $I_{\text{Ca-L}}$ -frequency relationship.

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The positive force–frequency relationship plays an important adaptive role in regulating heart function allowing appropriate response to the haemodynamic needs of the body. The positive relationship between the magnitude of L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca-L}}$ ) and frequency (use-dependent facilitation of  $I_{\text{Ca-L}}$ ) is an important part of this adaptive response. The mechanism of use-dependent facilitation of  $I_{\text{Ca-L}}$  was generally believed to relate to activation of calmodulin kinase (CaMKII). Evidence supporting this mechanism includes: (1) the fact that facilitation is largely eliminated by CaMKII blockers including KN-62 (Yuan & Bers, 1994), KN-93 (Maier *et al.* 2003) and various inhibitory peptides including CaMKII-209-390, CaMKII-273-302 (Yuan & Bers, 1994), ICK (Xiao *et al.* 1994) and AC3-I (Wu *et al.* 2001); and (2) that treatment with constitutively active CaMKII resulted in increases in open probability and prolonged open times of the L-type  $\text{Ca}^{2+}$  channel (Dzhura *et al.* 2000). Alternatively, we and others (Delgado *et al.* 1999; Guo & Duff, 2003) have provided evidence that sarcoplasmic reticulum (SR) calcium release negatively feeds back on

the calcium channel by causing inactivation as a major mechanism of use-dependent facilitation of  $I_{\text{Ca-L}}$ . In those studies, abrupt shortening of the stimulus cycle length significantly decreased SR  $\text{Ca}^{2+}$  release and thereby produced less inactivation, thus eliciting facilitation of  $I_{\text{Ca-L}}$  (Delgado *et al.* 1999; Guo & Duff, 2003). In the present study we clarify the role of endogenous CaMKII in use-dependent facilitation and more generally, in the frequency response of  $I_{\text{Ca-L}}$  in ventricular myocytes.

Recovery from inactivation plays an important role in the response of  $I_{\text{Ca-L}}$  to changes in pacing frequency. Slowing recovery from inactivation is expected to have an exaggerated effect on the  $I_{\text{Ca-L}}$  amplitude when pacing at high frequency. Vinogradova *et al.* 2000 reported that KN-93, as well as autocamtide-2 relating inhibitory peptide (AIP), slowed the recovery of  $I_{\text{Ca-L}}$  from inactivation in sinoatrial node cells of heart. In contrast, Yuan & Bers (1994) found that CaMK inhibitory peptide CaMKII-290-309 had no effect on  $I_{\text{Ca-L}}$  recovery in working cardiac myocytes. When applying the more selective, potent and cell membrane-permeable CaMKII

inhibitory peptide (myristoylated AIP), we observed that increases in  $[Ca^{2+}]_i$  produced by high-frequency pacing slowed recovery from inactivation. These data indicate that the  $Ca^{2+}$ -dependent activity of endogenous CaMKII accelerates recovery from inactivation and thus offsets the 'direct' effect of  $[Ca^{2+}]_i$  to slow recovery from inactivation.

## Methods

### Cell isolation

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996). The protocol was approved by the University of Calgary Animal Care Committee. Single ventricular myocytes were isolated from adult rat hearts using a Langendorff procedure similar to that used in our previous experiments (Guo & Duff, 2003). Adult, male, LBN rats (weight, 200–300 g) were deeply anaesthetized with  $CO_2$  and the heart was rapidly removed. The aorta was cannulated and retrograde perfusion was initiated with a standard Tyrode solution at  $37^\circ C$ . Perfusate was changed to a nominally  $Ca^{2+}$ -free Tyrode solution for 5 min followed by an 8-min perfusion with Tyrode solution containing  $10 \mu mol l^{-1} Ca^{2+}$  and  $0.05 mg ml^{-1}$  collagenase (Yakult, Tokyo, Japan). The left ventricle was removed and minced. Pieces of ventricle were gently agitated for 10–30 min in a shaking bath in the  $Ca^{2+}$ -free Tyrode solution containing  $0.5 mg ml^{-1}$  collagenase,  $0.1 mg ml^{-1}$  protease (type XII, Sigma, St Louis, MO, USA) and  $1 mg ml^{-1}$  bovine serum albumin. Aliquots of minced tissue were then drawn off at 5-min intervals, placed into Tyrode solution containing  $0.1 mmol l^{-1} Ca^{2+}$  and stored at room temperature ( $20^\circ C$  to  $22^\circ C$ ) until the myocytes were used. Single quiescent cardiomyocytes with smooth surfaces and clear cross-striation were used.

### Patch-clamp recordings

Conventional and perforated whole-cell voltage-clamp methods were used. All the experiments were performed at room temperature. Data acquisition and analysis were carried out using pCLAMP 8 (Axon Instruments, Inc., Union City, CA, USA) and SigmaPlot 9 (SPSS Inc., Chicago, IL, USA). An Axopatch 200B (Axon Instruments) amplifier was used. Access resistance was  $< 8 M\Omega$  in conventional whole-cell experiments. The  $\beta$ -escin perforated patch method (Fan & Palade, 1998) was also used in the present study. This method has been reported to reduce the  $I_{Ca-L}$  rundown (Fan & Palade, 1998). In this experiment,  $50 \mu mol l^{-1} \beta$ -escin was added into the pipette solution and the experiment was carried out only when the access resistance was less than  $15 M\Omega$ . In

all the experiments, the series resistance was routinely compensated to  $> 80\%$ .

To block  $K^+$  conductance, the extracellular solution was  $K^+$ -free and a  $Cs^+$ -containing pipette solution was used. Most of the  $Na^+$  current was blocked by  $20 \mu mol l^{-1}$  TTX. Any residual  $Na^+$  current and/or T-type  $Ca^{2+}$  current was inactivated by a ramp prepulse, which first stepped from  $-80$  to  $-60$  mV followed by a ramp to  $-40$  mV over 80 ms that was maintained at  $-40$  mV for another 20 ms.  $I_{Ca-L}$  was recorded using a depolarization test pulse to 0 mV from a holding potential of  $-80$  mV after the prepulse (Guo & Duff, 2003). The typical  $I_{Ca-L}$  recording pulse protocol was repeated at different frequencies or intervals according to the needs of experiments. The peak  $I_{Ca-L}$  at 0 mV was measured as the amplitude of  $I_{Ca-L}$ .

The time course of recovery from inactivation of  $I_{Ca-L}$  was measured using a conventional paired pulse protocol. The  $I_1-I_2$  intervals (the intervals between the first pulse and the second pulse) were systematically varied and relative recovery ratio was calculated as:

$$\text{Recovery ratio} = (I_{2,\text{peak}} - I_{1,\text{late}}) / (I_{1,\text{peak}} - I_{1,\text{late}})$$

where  $I_{\text{peak}}$  is the peak current level at the test pulse and  $I_{\text{late}}$  is the current level at the end of the test pulse. Most protocols were repeated every 10 s (0.1 Hz). To measure the time course of recovery of  $I_{Ca-L}$  when preceded by fast pacing, the 20 pulses at a frequency of 1 Hz were given before introduction of the first test paired pulse and five pulses at 1 Hz were given before each  $I_1-I_2$  pair (see Fig. 3A for protocol). All the recovery time courses were measured under the perforated patch-clamp configuration to prevent current rundown.

To allow comparison of the recovery time course between slow (0.1 Hz) and fast pacing (1 Hz), the recovery ratio during fast pacing was corrected using the following formula:

$$\text{Recovery ratio} = \frac{\text{Recovery ratio } (I_2/I_1)}{\text{Recovery ratio } (I_{10s}/I_1)}$$

where recovery ratio ( $I_{10s}/I_1$ ) is the recovery ratio calculated by traditional formula described above, when the  $I_1-I_2$  interval was increased to 10 s during the fast pacing protocols. Although the  $\beta$ -escin perforated-patch technique was used, a small change of  $I_{Ca-L}$  with time (rundown or runup) still can be seen. We carefully monitored amplitude of all  $I_1$  values. If the amplitude of adjacent  $I_1$  (the  $I_1$  before and after every  $I_2$ ) was significantly altered, the data were discarded.

### Solution and drugs

The Tyrode solution contained ( $mmol l^{-1}$ ): NaCl 145, KCl 5.4,  $CaCl_2$  1.8,  $MgCl_2$  1.0,  $Na_2HPO_4$  1.0, Hepes 5.0 and glucose 5.5; pH adjusted to 7.4 with NaOH.

Unless otherwise indicated, the extracellular superfusate contained  $20 \mu\text{mol l}^{-1}$  TTX and was  $\text{K}^+$ -free, with  $\text{K}^+$  being replaced by equimolar  $\text{Cs}^+$ . The pipette solution contained ( $\text{mmol l}^{-1}$ ): CsCl 120, tetraethylammonium chloride 20, ATP-Mg 5, GTP- $\text{Na}_2$  0.5, EGTA 0.2, Hepes 10; pH adjusted to 7.2 with CsOH. For the experiments,  $5 \text{ mmol l}^{-1}$  EGTA and  $2 \text{ mmol l}^{-1}$   $\text{CaCl}_2$ , or  $5 \text{ mmol l}^{-1}$  BAPTA and  $1.36 \text{ mmol l}^{-1}$   $\text{CaCl}_2$  (the calculated free  $[\text{Ca}^{2+}]$  in the pipette solution was  $\sim 100 \text{ nmol l}^{-1}$  (Bers *et al.* 1994)) were added into pipette solutions according to the requirements.

EGTA was added to the pipette solution in some  $\beta$ -escin perforated patch-clamp experiments. EGTA passes through the  $\beta$ -escin channels (Fan & Palade 1998). The effect of EGTA dialysis was confirmed by the observation that cell shortening ceased when recording  $I_{\text{Ca-L}}$ . For the BAPTA experiments, the cells were incubated in  $20 \mu\text{mol l}^{-1}$  BAPTA-AM for more than 1 h before the experiment, and  $5 \text{ mmol l}^{-1}$  BAPTA was added into the pipette solution during the experiment.

For AIP experiments,  $2 \mu\text{mol l}^{-1}$  membrane permeable AIP (myristoylated AIP) was added to the recording solution and cells were preincubated with AIP in the Tyrode solution contain  $1 \text{ mmol l}^{-1}$   $\text{CaCl}_2$  for more than 30 min before beginning the experiments. In a few cells, AIP was dialysed through the patch pipette. In this case,  $100 \mu\text{mol l}^{-1}$  AIP (not myristoylated AIP) was added into the pipette solution. To enhance dialysis, a brief positive pressure was applied to the patch pipette during which the access resistance was less than  $5 \text{ M}\Omega$ . No difference in AIP response was found when comparing membrane permeable AIP with dialysis and accordingly, we added the data sets together.

When required, cells were incubated in  $1 \mu\text{mol l}^{-1}$  thapsigargin for more than 1 h before experiments. AIP, myristoylated AIP, KN-93, KN-92, TTX and thapsigargin were obtained from Calbiochem (CA, USA). All other chemicals were acquired from Sigma.

### Data analysis

$I_{\text{Ca-L}}$  inactivation time ( $T_{0.37}$ ) was measured as the time required for the current to decay to 0.37 of the peak amplitude. Fifty percent recovery time ( $T_{0.5}$ ) of  $I_{\text{Ca-L}}$  from inactivation was measured as the  $I_1$ – $I_2$  intervals required for  $I_2$  to recover to 50% of  $I_1$ . Results are presented as means  $\pm$  s.e.m. Data were analysed with paired and unpaired *t* tests.

## Results

### AIP eliminates facilitation of $I_{\text{Ca-L}}$ but only at high-frequency pacing and without an effect on $I_{\text{Ca-L}}$ inactivation

To evaluate the effects of CaMKII on facilitation, rat cardiac myocytes were incubated for  $> 30$  min with

membrane-permeable AIP (myristoylated AIP) (or AIP in the pipette) and compared with the vehicle control. Frequency-dependent changes in  $I_{\text{Ca-L}}$  were evaluated during abrupt increases in pacing rate from 0.05 Hz to a range of cycle lengths from 0.5 to 2 Hz. A significant increase in  $I_{\text{Ca-L}}$  was observed at abrupt increases in pacing rate. AIP inhibited facilitation but it was surprising to find that the extent of inhibition was cycle-length dependent (Fig. 1A and B). At a pacing frequency of 2 Hz, AIP completely eliminated  $I_{\text{Ca-L}}$  facilitation after an abrupt increase of pacing frequency. Moreover, a progressive decrease of the currents occurred during continued fast pacing, suggesting an accumulative effect. This progressive decrease rapidly recovered to the baseline level after the pacing frequency was slowed (Fig. 1B). When pacing at 1 and 0.5 Hz, the effects of AIP were substantially less (Fig. 1B). Similar changes were observed in all five experiments with AIP. Figure 1C shows the mean frequency-dependent effect of AIP on facilitation. At 2 Hz, a significant decrease was observed but non-significant trends were seen at pacing frequencies of 1 and 0.5 Hz ( $n=7$  in control,  $n=5$  with AIP). During prolonged fast pacing, AIP completely inhibited the positive  $I_{\text{Ca-L}}$ –frequency relationship; however, this was not evident during slower pacing frequencies. It is interesting that the extent of inhibition of facilitation progressively increased during application of multiple fast stimuli after blocking endogenous CaMKII.

SR calcium release negatively feeds back on the calcium channel by producing and accelerating inactivation. Thapsigargin eliminates facilitation by decreasing the difference in the kinetics of onset of inactivation between the  $I_1$  and  $I_2$  pulses (Guo & Duff, 2003). In contrast, AIP inhibits facilitation at fast pacing frequency but without a change in the kinetics of inactivation between the  $I_1$  and  $I_2$  beats. The pattern of inactivation during AIP treatment was similar to control. During AIP treatment, while the magnitudes of  $I_1$  and  $I_2$  were similar, the kinetics of inactivation of the  $I_2$  pulse were still much slower than those of  $I_1$  (similar to control). AIP did not significantly change the inactivation time,  $T_{0.37}$ , at all tested pacing frequencies ( $n=5$ ) compared to control ( $n=7$ , Fig. 1D). This observation does not support the previous hypothesis that activation of CaMKII decreases  $I_{\text{Ca-L}}$  inactivation at high pacing frequency (Maier *et al.* 2003). It also makes it unlikely that AIP modulates the response of  $I_{\text{Ca-L}}$  to change in pacing frequency through its effects on SR  $\text{Ca}^{2+}$  storage and release.

### AIP slows recovery from inactivation of $I_{\text{Ca-L}}$

To completely remove the contribution of SR  $\text{Ca}^{2+}$  release on the recovery of  $I_{\text{Ca-L}}$  (Guo & Duff, 2003), myocytes were pretreated for  $> 1$  h with  $1 \mu\text{mol l}^{-1}$  thapsigargin. Facilitation is not manifest after thapsigargin treatment. The magnitude of change in  $I_{\text{Ca-L}}$  was assessed during

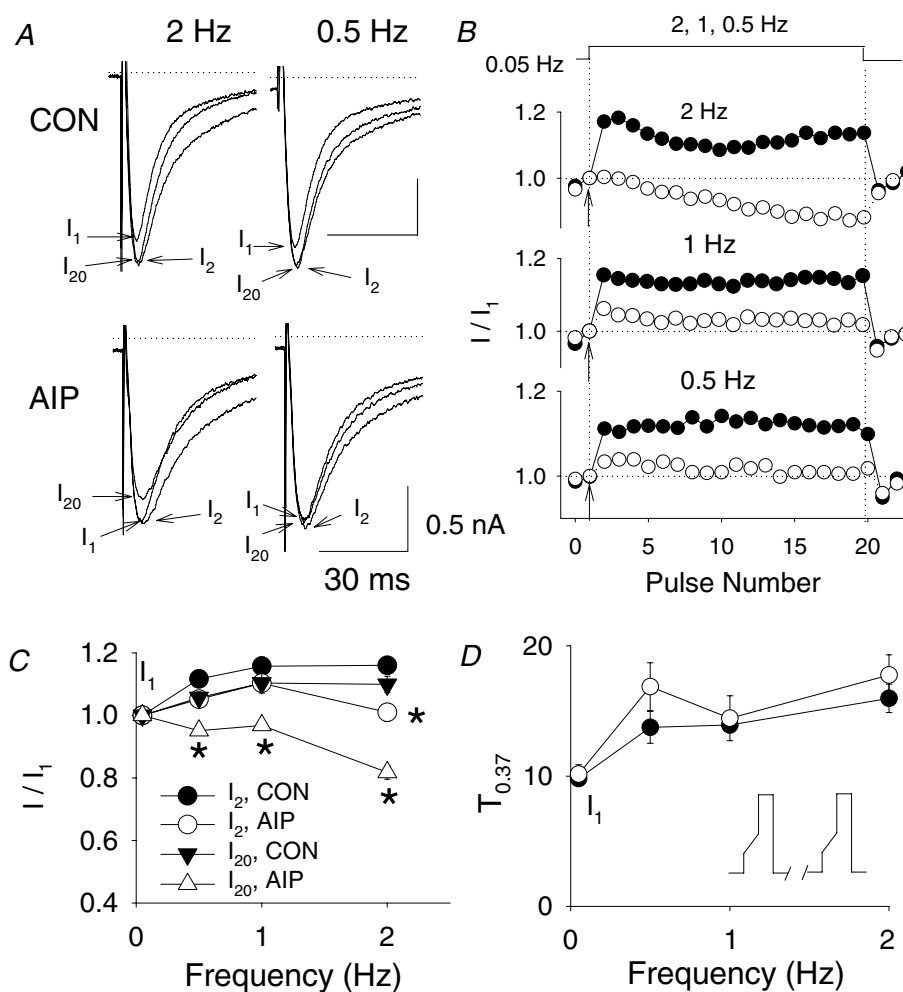
application of paired pulses at various  $I_1$ – $I_2$  coupling intervals (Fig. 2A). By 0.4 s,  $I_{Ca-L}$  had recovered almost to baseline ( $I_1$ ) under control conditions whereas in cells treated with AIP, the recovery had only reached about 70%. Figure 2B shows that AIP significantly prolonged the time course of recovery from inactivation. This slowing of recovery from inactivation occurs without any significant alteration of the time constant of onset of inactivation (Fig. 2C). To provide further evidence that slowing recovery from inactivation is due to inhibition of CaMKII, the effects of another CaMKII inhibitor, KN-93, were compared to the effects of its inactive analogue KN-92. Like AIP,  $2 \mu\text{mol l}^{-1}$  KN-93 significantly slowed recovery from inactivation, compared to the response with  $2 \mu\text{mol l}^{-1}$  KN-92 (Fig. 2D and E). It is interesting that KN-93 showed a much stronger inhibitory effect

on the  $I_{Ca-L}$  recovery, which suggests that KN-93 was a more complete inhibitor of CaMKII than AIP, under our experimental conditions.

Figure 2E shows that AIP increased the 50% recovery time ( $T_{0.5}$ ) of  $I_{Ca-L}$  from  $0.161 \pm 0.005$  s in control ( $n = 7$ ) to  $0.21 \pm 0.018$  s ( $n = 8$ ). Similarly, KN-93 increased  $T_{0.5}$  from  $0.133 \pm 0.004$  (in the presence of KN-92) to  $0.400 \pm 0.064$  s ( $n = 3$ ). These data indicate that CaMKII does significantly slow the  $I_{Ca-L}$  recovery from inactivation.

### The effect of AIP is dependent on cycle length

As high-frequency activation of  $\text{Ca}^{2+}$  transients mediates progressive increases in CaMKII activity (De Koninck & Schulman, 1998), it seems plausible that the effects of AIP



**Figure 1. AIP inhibits the use-dependent facilitation of  $I_{Ca-L}$  in a frequency-dependent manner**

A, representative examples are shown for the first ( $I_1$ ), second ( $I_2$ ) and 20th  $I_{Ca-L}$  ( $I_{20}$ ) traces after the pacing frequencies were abruptly increased from 0.05 to 0.5 and 2 Hz in control (CON) and in the presence of  $2 \mu\text{mol l}^{-1}$  AIP.  $I_{Ca-L}$  was induced by a depolarization to 0 mV from  $-80$  mV after a ramp prepulse. Arrows indicate the peaks of currents. B, normalized  $I_{Ca-L}$  amplitude during the pacing frequency changes in control (●) and the presence of AIP (○) obtained from the experiments shown in A. All current amplitudes were normalized to  $I_1$  (arrows). C, average  $I_2/I_1$ – and  $I_{20}/I_1$ –frequency relationship of  $I_{Ca-L}$  in control ( $n = 7$ ) and the presence of AIP ( $n = 5$ ). D, mean inactivation time,  $T_{0.37}$ , of  $I_2$  is plotted against the pacing frequency, showing control and with AIP. The experimental protocol is shown in the inset. \* $P < 0.01$ .

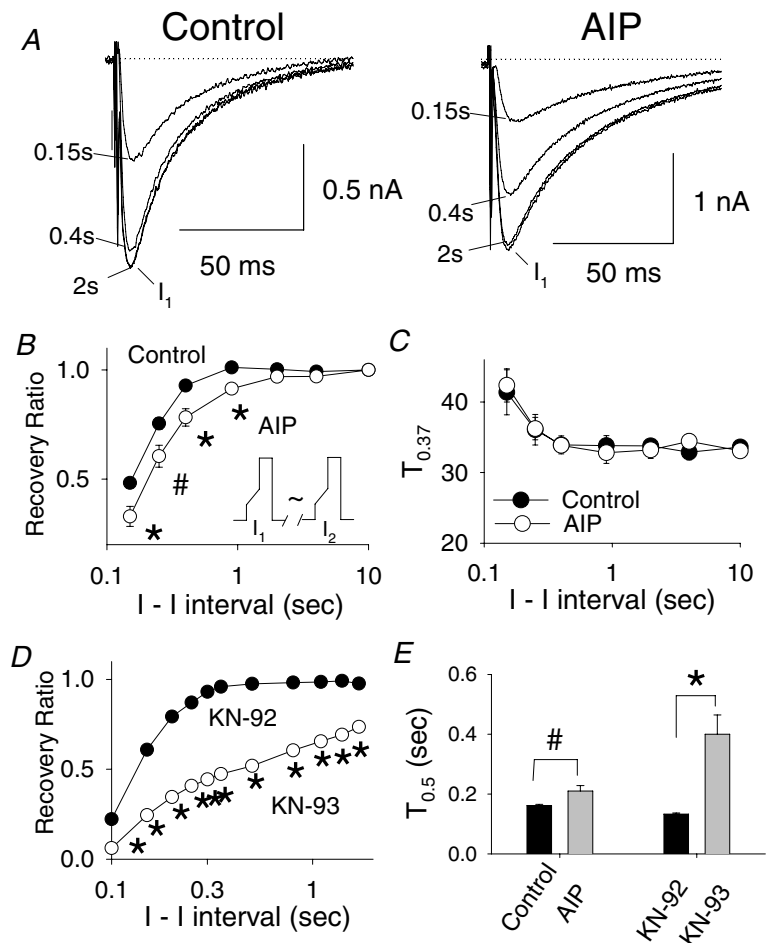
would be dependent on cycle length. After the contribution of SR Ca<sup>2+</sup> stores and release had been eliminated by pretreatment with 1 μmol l<sup>-1</sup> thapsigargin, the time course of recovery from inactivation of I<sub>Ca-L</sub> was measured by application of paired pulses at various I<sub>1</sub>-I<sub>2</sub> intervals. To assess the recovery of inactivation when pacing at high frequency, the impact of application of 20 pulses at a frequency of 1 Hz was evaluated. Figure 3A shows the time course of recovery from inactivation when preconditioned at a fast (1 Hz) compared with a slow (0.1 Hz) pacing frequency, with or without pretreatment with AIP. When no drug was present, preconditioning did not change recovery of I<sub>Ca-L</sub> (Fig. 3A; slow pacing, ●; fast pacing, ▼; n = 7 and 10, respectively) independent of whether preconditioned by fast or slow pacing; in fact, fast pacing trended to slow recovery. However, after blocking endogenous CaMKII with 2 μmol l<sup>-1</sup> AIP, preconditioning at fast pacing rates significantly slowed recovery from inactivation (n = 11, 0.1 Hz, ○; 1.0 Hz, △ in Fig. 3A). The mean recovery time courses (T<sub>0.5</sub>) are shown in the inset of Fig. 3A.

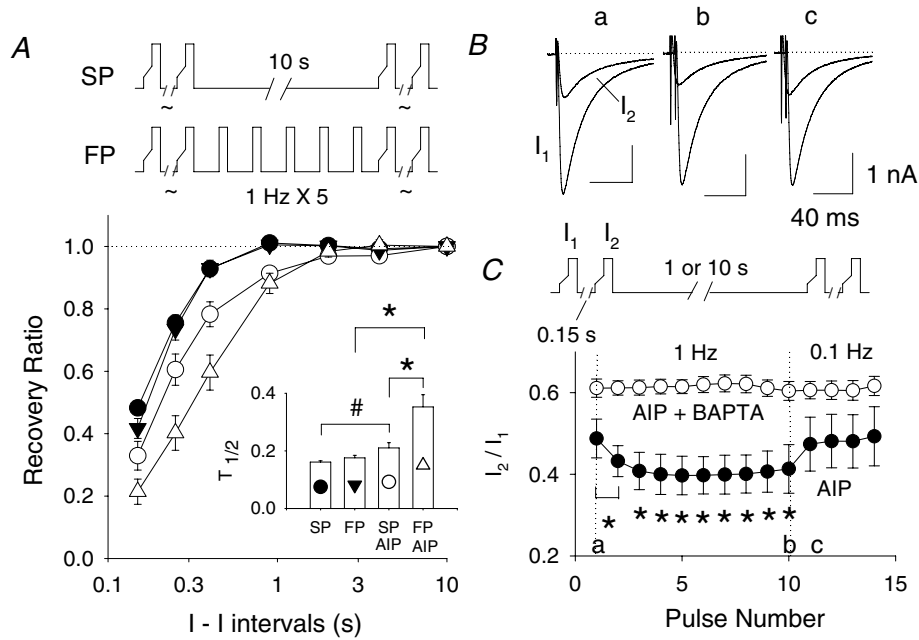
To address whether changes in intracellular Ca<sup>2+</sup> mediate the effects of the fast preconditioning pulse trains, we compared the effects of the preconditioning trains

on I<sub>2</sub>/I<sub>1</sub> recovery in the presence or absence of BAPTA. The protocol shown on top of Fig. 3C was used. Paired pulses at a fixed I<sub>1</sub>-I<sub>2</sub> interval (0.15 s) were applied but the interpulse interval was either 10 or 1 s. Two transitions were examined. After more than a 10-s rest, the interpulse interval was increased to 1 s and then it was slowed to 10 s (protocol shown in inset to Fig. 3C). The dynamics of the effect of changes in interpulse interval during the transitions are plotted. During blockade of endogenous CaMKII with AIP alone, abrupt increases in pacing frequency decreased the I<sub>2</sub>/I<sub>1</sub> ratios, which then returned to baseline when pacing frequency slowed (0.1 Hz; Fig. 3B and C). It is important to note that the decrease of I<sub>2</sub>/I<sub>1</sub> ratio in response to fast pacing was completely eliminated by treatment with BAPTA. These data indicate that in the absence of endogenous CaMKII activity, fast pacing slows recovery from inactivation and that this effect is probably mediated by changes in [Ca<sup>2+</sup>]<sub>i</sub>.

**Intracellular Ca<sup>2+</sup> directly inhibits the recovery from inactivation of I<sub>Ca-L</sub>**

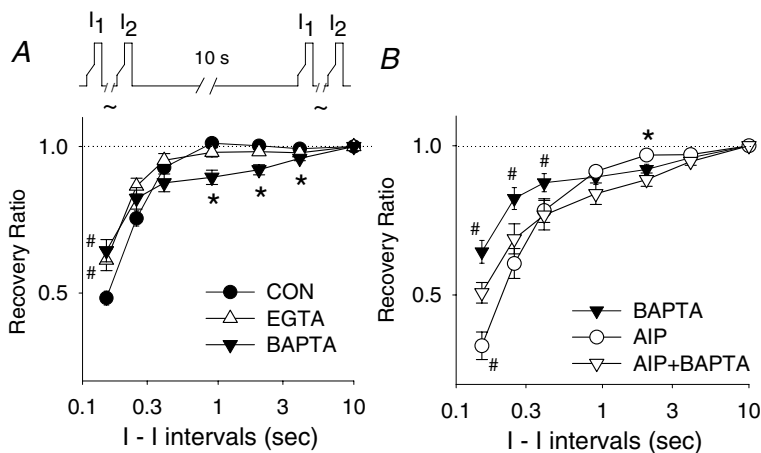
To provide further evidence that [Ca<sup>2+</sup>]<sub>i</sub> is an important determinant of recovery from inactivation, the effect of





two  $Ca^{2+}$  chelators, BAPTA and EGTA, dialysed intracellularly, were evaluated. BAPTA chelates  $Ca^{2+}$  more rapidly than EGTA (Tsien, 1980). Cells were pretreated with thapsigargin to eliminate any contribution of SR  $Ca^{2+}$  stores or release on recovery from inactivation. Figure 4A shows that BAPTA had a biphasic effect: at short  $I_1$ – $I_2$  intervals ( $< 0.5$  s), BAPTA accelerated recovery

from inactivation; whereas at long coupling intervals ( $I_1$ – $I_2$  intervals longer than 0.5 s), it slowed recovery (Fig. 4A). Both effects were statistically significant ( $n = 10$  in control,  $n = 7$  with BAPTA). In contrast, EGTA, a slow  $Ca^{2+}$  chelator (Tsien, 1980), accelerated the recovery from inactivation at short  $I_1$ – $I_2$  coupling intervals and had no effect at longer intervals. These data indicate that BAPTA



appears to have two effects, one acting directly and one acting through CaMKII.

To further explore this duality, we assessed the effects of AIP ( $2 \mu\text{mol l}^{-1}$ ) in the presence of BAPTA (Fig. 4B). BAPTA was expected to chelate free  $\text{Ca}^{2+}$  rapidly and largely prevent the activation of CaMKII and for the most part eliminate the AIP effect. However, the  $I_{\text{Ca-L}}$  recovery in the presence of AIP plus BAPTA was significantly slower than that observed with BAPTA alone. These data suggests that BAPTA did not completely eliminate endogenous CaMKII activity.

Previous studies indicate that CaMKII maintains its activity long after a  $\text{Ca}^{2+}$  transient through auto-phosphorylation. The effect of BAPTA to slow recovery of  $I_{\text{Ca-L}}$  at longer coupling intervals may represent its ability to block CaMKII. If AIP could completely inhibit CaMKII activity, the effect of BAPTA on recovery at long coupling intervals would be expected to disappear. However, Fig. 4B shows that after blocking endogenous CaMKII with  $2 \mu\text{mol l}^{-1}$  AIP, BAPTA still slowed the recovery at long coupling intervals ( $I_1-I_2 > 0.5 \text{ s}$ ,  $P < 0.01$  at 2 s, BAPTA plus AIP) when comparing with AIP alone. Thus, assuming this is due to the effects of BAPTA on CaMKII activity, AIP may not be completely inhibiting CaMKII under these conditions.

## Discussion

A novel  $\text{Ca}^{2+}$ -CaMKII regulatory pathway is proposed by this study: intracellular  $\text{Ca}^{2+}$  directly inhibits the  $I_{\text{Ca-L}}$  recovery from inactivation, while  $\text{Ca}^{+}$ -dependent CaMKII activity facilitates recovery from inactivation. Under physiological conditions of  $[\text{Ca}^{2+}]_i$  oscillations, endogenous CaMKII activity compensates for the direct inhibitory effect of  $[\text{Ca}^{2+}]_i$  on  $I_{\text{Ca-L}}$  recovery from inactivation. The presence of the new regulatory pathway is supported by the following evidence. (1) The CaMKII inhibitory peptide, AIP, eliminates the cycle length-dependent adaptive increase in  $I_{\text{Ca-L}}$  after abrupt increase in pacing frequency. This effect is exaggerated at fast pacing frequency. (2) After eliminating SR  $\text{Ca}^{2+}$  release, AIP, as well as KN-93, significantly slows  $I_{\text{Ca-L}}$  recovery from inactivation. (3) In the presence of thapsigargin, high-frequency pacing exaggerates the inhibitory effects of AIP on recovery from inactivation, and high-frequency pacing has no effect in the absence of AIP. (4) Buffering of intracellular  $\text{Ca}^{2+}$  substantially accelerates the  $I_{\text{Ca-L}}$  recovery from inactivation and eliminates the inhibitory effect of high-frequency pacing on recovery during AIP treatment.

### $I_{\text{Ca-L}}$ recovery is facilitated by endogenous CaMKII

Our previous study (Guo & Duff, 2003) showed that SR  $\text{Ca}^{2+}$  release acting through  $\text{Ca}^{2+}$ -dependent inactivation

has an important effect on the amplitude of  $I_{\text{Ca-L}}$  especially when two tightly coupled pulses are applied. Thus, when the SR function is intact, the recovery course of  $I_{\text{Ca-L}}$  inactivation will be dominantly affected by an SR  $\text{Ca}^{2+}$ -related mechanism. Probably this is one of the main reasons that the effect of CaMKII on  $I_{\text{Ca-L}}$  recovery from inactivation has not been previously emphasized in working ventricular myocytes. In sinoatrial node cells, KN-93 as well as AIP slowed the  $I_{\text{Ca-L}}$  recovery (Vinogradova *et al.* 2000). A less developed SR  $\text{Ca}^{2+}$  store system in sinoatrial node cells probably favoured this observation. In working ventricular myocytes, some strong but less specific CaMK inhibitors, KN-62 and the less selective inhibitor, ruthenium red, have been reported to inhibit recovery from inactivation of  $I_{\text{Ca-L}}$  (Yuan & Bers, 1994; Sanchez *et al.* 2001; Netticadan *et al.* 1996). Probably due to the strong influence of the negative feedback of SR  $\text{Ca}^{2+}$  on  $I_{\text{Ca-L}}$ , Yuan & Bers (1994) could not reproduce these data using more selective inhibitory peptides of CaMKII in ventricular myocytes.

By eliminating the dominant regulatory factor of SR  $\text{Ca}^{2+}$  release (thapsigargin) and by using high-frequency pacing to increase endogenous CaMKII activity, we have shown clearly in this study a significant slowing of  $I_{\text{Ca-L}}$  recovery from inactivation by inhibition of endogenous CaMKII in the ventricular myocytes. It is important to note that this effect is  $\text{Ca}^{2+}$ -dependent (Fig. 3B and C). AIP has its greatest effect during fast pacing, a condition that will increase endogenous CaMKII activity through elevated  $\text{Ca}^{2+}$  concentrations (De Koninck & Schulman, 1998).

In this study, KN-93 showed a much stronger inhibitory effect than AIP on  $I_{\text{Ca-L}}$  recovery. The difference may be explained by more complete inhibition of CaMKII by KN-93. AIP at  $2 \mu\text{mol l}^{-1}$  probably did not completely inhibit endogenous CaMKII in our study. However, KN-93 inhibits many subtypes of calmodulin kinases, whereas AIP is more specific for CaMKII (Ishida *et al.* 1995). In Fig. 4B, at  $I_1-I_2$  intervals of  $> 0.5 \text{ s}$ , AIP plus BAPTA showed slower  $I_{\text{Ca-L}}$  recovery than did AIP alone. The cell permeability of myristoylated AIP may be the rate limiting step. Investigators have used a wide variety of myristoylated AIP concentrations from 1 to  $20 \mu\text{mol l}^{-1}$  (Gailly, 1998; Tong *et al.* 2004; Vinogradova *et al.* 2000).

### Intracellular $\text{Ca}^{2+}$ directly inhibits $I_{\text{Ca-L}}$ recovery

The present study shows that after inhibition of endogenous CaMKII activity,  $I_{\text{Ca-L}}$  recovery is significantly slowed by increasing  $[\text{Ca}^{2+}]_i$  at fast pacing rates (De Koninck & Schulman, 1998) and recovery is accelerated by chelation of  $\text{Ca}^{2+}$  by BAPTA and EGTA. This may reflect a direct effect of  $[\text{Ca}^{2+}]_i$  on the dynamics of dissociation of  $\text{Ca}^{2+}$  from the inactivation gate. Under physiological conditions, the direct inhibitory effect is

largely overcome by the opposing effect of  $\text{Ca}^{2+}$ -dependent activation of CaMKII. The recovery from the inactivation of  $I_{\text{Ca(v)1.2}}$  expressed in HEK cells has been evaluated at various  $[\text{Ca}^{2+}]_i$  by Lacinova & Hofmann (2005). In that study, increased  $[\text{Ca}^{2+}]_i$  slowed recovery from the inactivation of  $I_{\text{Ca(v)1.2}}$ . CaMKII was not blocked or evaluated in their experiments. In HEK cells, the endogenous CaMKII activity will not be spatially or physiologically matched to the level of expression of Ca(v)1.2. Thus, the imbalance of endogenous CaMKII activity and  $I_{\text{Ca(v)1.2}}$  expression in HEK cells would allow them to observe the direct effect of  $[\text{Ca}^{2+}]_i$  on recovery but would not allow them to uncover the complex regulatory pathway described herein.

### Functional importance of accelerated recovery from inactivation by CaMKII activity

In the present study, we evaluated the effects of endogenous CaMKII in the presence of thapsigargin to eliminate SR  $\text{Ca}^{2+}$  release. However, under physiological conditions, SR  $\text{Ca}^{2+}$  release would be expected to contribute  $\sim 90\%$  of the systolic  $\text{Ca}^{2+}$  transient (Bers, 2001). Thus, it is reasonable to expect that under physiological conditions, the SR  $\text{Ca}^{2+}$  release would amplify both the direct and indirect regulatory mechanisms. The effects will be further exaggerated at fast pacing rates. Therefore, we propose that most of the effects of AIP on the frequency-dependent changes of  $I_{\text{Ca-L}}$  (Fig. 1) are attributable to the recovery from the inactivation mechanism.

### Relationship to previous proposed regulatory mechanisms

Previous studies reported that CaMKII facilitates L-type  $\text{Ca}^{2+}$  channel activity by a direct interaction with the channel (Hudmon *et al.* 2005; Dzhura *et al.* 2000). After eliminating  $\text{Ca}^{2+}$ -dependent inactivation of L-type channel by mutation, endogenous CaMKII activity was responsible for increases in the amplitude of  $I_{\text{Ca-L}}$  after abrupt increases in pacing frequency (Hudmon *et al.* 2005). Constitutively active CaMKII increased the open probability and prolonged the open times of the L-type  $\text{Ca}^{2+}$  channel when  $\text{Ba}^{2+}$  was used as a charge carrier (Dzhura *et al.* 2000). Thus  $\text{Ca}^{2+}$ -dependent inactivation and recovery from inactivation of  $I_{\text{Ca-L}}$  were not involved in their observations. Theoretically, a direct interaction of  $\text{Ca}^{2+}$ -CaMKII with the channel could accelerate recovery from inactivation. In the present study, we intermittently observed possible direct facilitation (recovery ratio of  $> 1$  in the presence of thapsigargin) but the magnitude of this facilitation was quite small. At the point of its greatest magnitude, at an  $I-I$  interval of 0.9 s, the recovery ratios were  $1.011 \pm 0.010$  and  $1.006 \pm 0.015$ , at slow and fast

pacing rates, respectively (Fig. 3). Thus, it is unlikely that the direct facilitation mechanism of  $\text{Ca}^{2+}$ -CaMKII could exclusively explain the effect of CaMKII on the recovery from inactivation observed in this study. After inhibition of CaMKII by AIP and KN-93, the  $T_{0.5}$  almost doubled (Figs 2 and 3). We believe that CaMKII accelerates the recovery from inactivation independent of its previously published direct mechanism.

### Effects of BAPTA on recovery from inactivation

Binding of  $\text{Ca}^{2+}$ -calmodulin to CaMKII activates it. BAPTA and EGTA chelate free  $\text{Ca}^{2+}$  and thus compete with CaMKII for  $\text{Ca}^{2+}$ -calmodulin binding. BAPTA and EGTA inhibit endogenous CaMKII activity. In this study, BAPTA accelerates the recovery from inactivation at tight coupling intervals ( $I-I$  interval  $< 0.5$  s; Fig. 4B) but slows the recovery at longer coupling intervals ( $I-I$  intervals  $> 0.5$  s). This finding may be explained by a dual physiological effect of BAPTA: (1) it chelates intracellular  $\text{Ca}^{2+}$  thereby inhibiting its direct effect to slow recovery; and (2) it inhibits  $\text{Ca}^{2+}$ -dependent activation of CaMKII activity thus producing the opposite effect. After  $\text{Ca}^{2+}$  activation, CaMKII autophosphorylates and maintains its activity even after  $[\text{Ca}^{2+}]_i$  decays (De Koninck & Schulman, 1998). The ability of BAPTA to slow the recovery at long coupling intervals may reflect its ability to eliminate the autophosphorylated CaMKII effect. In contrast, the ability of BAPTA to accelerate recovery from inactivation at tight coupling intervals may reflect inhibition of the direct effect of  $\text{Ca}^{2+}$ .

Use-dependent facilitation can be eliminated by treatment with thapsigargin or ryanodine. Previous data (Wu *et al.* 1999; Anderson, 2004), have been interpreted to indicate that  $\text{Ca}^{2+}$  released by the SR rather than direct flux of  $\text{Ca}^{2+}$  entering through the pore of the L-type calcium channel activates CaMKII. However, recently it was suggested that the flux of Ca through the pore could activate CaMKII and thus modulate the function of L-type channel (Hudmon *et al.* 2005). Our study suggests that the CaMKII activity, which accelerates recovery from inactivation, is sensitive to local  $\text{Ca}^{2+}$  released from the inner mouth of the pore of the channel because BAPTA cannot completely eliminate the effect of AIP. Similarly, BAPTA cannot completely eliminate  $\text{Ca}^{2+}$ -dependent inactivation of  $I_{\text{Ca-L}}$  (Kreiner & Lee, 2006). The  $\text{Ca}^{2+}$ -binding site for the inactivation gate has been considered to be near or within the internal mouth of L-type  $\text{Ca}^{2+}$  channel (Naraghi & Neher, 1997).

Other studies also suggest a similar location for sites regulating CaMKII binding and sites mediating  $\text{Ca}^{2+}$ -dependent inactivation. Binding of  $\text{Ca}^{2+}$ -calmodulin to the C-terminal of the L-type  $\text{Ca}^{2+}$  channel has been proposed as a sensor for  $\text{Ca}^{2+}$ -dependent inactivation (Alseikhan *et al.* 2002; Peterson *et al.* 1999; Pitt *et al.*



2001; Zuhlke *et al.* 1999, Zuhlke *et al.* 2000). Similarly, the binding site for CaMKII is proposed to exist in the C-terminal of the L-type  $\text{Ca}^{2+}$  channel (Hudmon *et al.* 2005). Dzhura *et al.* (2000) and Wu *et al.* (2001) have reported that calmodulin kinase and a calmodulin-binding 'IQ' domain both facilitate L-type  $\text{Ca}^{2+}$  current in rabbit ventricular myocytes via a similar mechanism. Cytoskeletal disrupting agents also prevent calmodulin kinase, IQ domain and voltage-dependent facilitation of L-type  $\text{Ca}^{2+}$  channels (Dzhura *et al.* 2002).

Kinetically, EGTA is a much slower  $\text{Ca}^{2+}$  buffer than BAPTA (~100 times slower; Tsien, 1980). In our study, EGTA was not able to inhibit recovery at long coupling intervals (Fig. 4A). This suggests that EGTA may not effectively inhibit endogenous CaMKII activity. A similar difference between BAPTA and EGTA on their ability to inhibit endogenous CaMKII activity has been reported in cardiac sinoatrial node cells (Vinogradova *et al.* 2000). A possible explanation may relate to the relative affinity and kinetics of binding between endogenous CaMKII, EGTA and BAPTA-AM. Our data could be explained if: (1) CaMKII binds  $\text{Ca}^{2+}$  faster than EGTA but slower than BAPTA; (2) the subsarcolemmal compartment in proximity to the  $\text{Ca}^{2+}$  channel is more accessible to BAPTA than EGTA; or (3) insufficient EGTA entered the cell by dialysis. Against the latter possibility, EGTA effectively inhibited myocyte shortening. EGTA is traditionally and routinely added to the pipette solution for patch-clamp experiments when  $\text{Ca}^{2+}$  chelation is required. Different results may be obtained depending on the intracellular  $\text{Ca}^{2+}$  ion chelator used. Based on our study, if EGTA is used to chelate intracellular  $\text{Ca}^{2+}$ , CaMKII activity may be maintained and recovery of  $I_{\text{Ca-L}}$  may be accelerated.

We propose a novel regulatory pathway which involves opposing effects of  $[\text{Ca}^{2+}]_i$  on recovery from inactivation; a direct effect to slow and a  $\text{Ca}^{2+}$ -dependent CaMKII effect to accelerate recovery from inactivation. CaMKII is activated by  $[\text{Ca}^{2+}]_i$  and acts as a compensatory mechanism to respond to cycle length changes via phosphorylation and autophosphorylation of CaMKII. Any changes in CaMKII activity will change this balance and produce significant changes in  $I_{\text{Ca-L}}$ ,  $[\text{Ca}^{2+}]_i$  and cellular function. Thus, this novel regulatory pathway is likely to be an important determinant of response to physiological and pathological stimuli.

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